Exhibit C

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Biochemistry

A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: Cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells

(genotypes/immune response/vaccination/chronic hepatitis)

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ABSTRACT **Hepatitis C virus (HCV) is a major cause of chronic hepatitis. The virus does not replicate efficiently in cell cultures, and it is therefore difficult to assess infection-neutralizing antibodies and to evaluate protective immunity *in vitro*. To study the binding of the HCV envelope to cell-surface receptors, we developed an assay to assess specific binding of recombinant envelope proteins to human cells and neutralization thereof. HCV recombinant envelope proteins expressed in various systems were incubated with human cells, and binding was assessed by flow cytometry using anti-envelope antibodies. Envelope glycoprotein 2 (E2) expressed in mammalian cells, but not in yeast or insect cells, binds human cells with high affinity ($K_d \sim 10^{-8}$ M). We then assessed antibodies able to neutralize E2 binding in the sera of both vaccinated and carrier chimpanzees, as well as in the sera of humans infected with various HCV genotypes. Vaccination with recombinant envelope proteins expressed in mammalian cells elicited high titers of neutralizing antibodies that correlated with protection from HCV challenge. HCV infection does not elicit neutralizing antibodies in most chimpanzees and humans, although low titers of neutralizing antibodies were detectable in a minority of infections. The ability to neutralize binding of E2 derived from the HCV-1 genotype was equally distributed among sera from patients infected with HCV genotypes 1, 2, and 3, demonstrating that binding of E2 is partly independent of E2 hypervariable regions. However, a mouse monoclonal antibody raised against the E2 hypervariable region 1 can partially neutralize binding of E2, indicating that at least two neutralizing epitopes, one of which is hypervariable, should exist on the E2 protein. The neutralization-of-binding assay described will be useful to study protective immunity to HCV infection and for vaccine development.**

Hepatitis C virus (HCV) has become a major health problem: 0.4–1.5% of the population worldwide is chronically infected (1). Infection is mostly asymptomatic, most of the infected individuals became lifelong carriers (2), and chronic hepatitis develops in ≈50% of these individuals (3).

HCV is a positive-sense RNA virus of about 10,000 nt, with a single open reading frame encoding for a polyprotein of ≈3000 amino acids (4). The functions of viral proteins produced by proteolytic cleavage of the polyprotein have been inferred by analogy with known viruses of similar genomic organization. Two glycoproteins, named E1 and E2, have been suggested to be the external proteins of the viral envelope (5) that are responsible for binding of the virus to target cells.

A first step in designing an HCV vaccine is identification of the components involved in protective immunity. At present, little is known of the role the immune response plays in the course of HCV infection. In a passive immunization study in chimpanzees, HCV infection has been prevented after *in vitro* neutralization with plasma of a chronically infected patient (6). However, the assessment of protective antibody responses to HCV has been hampered by the absence of an efficient neutralization assay *in vitro*. Because HCV does not grow efficiently in cell cultures, attempts have been made (7, 8) to set up neutralization tests that estimated HCV binding to target cells. However, the available tests are based on the detection of bound virus by PCR and have obvious shortcomings, such as the difficulties in quantitating neutralizing antibodies and problems in obtaining accurate reproduction of PCR testing.

Here we describe a quantitative test [named neutralization of binding (NOB)] estimating HCV neutralizing antibodies, which is based on the cytofluorimetric assessment of sera that neutralize the binding of HCV envelope glycoprotein 2 (E2) to human cells.

MATERIALS AND METHODS

Cells. The human T-cell lymphoma line, MOLT-4, was obtained from ATCC (Rockville, MD). Cells were grown in RPMI 1640 (GIBCO) medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (GIBCO).

Recombinant Envelope Proteins. The glycoproteins E1/$E2_{192-746}$ were expressed in HeLa or CHO cells, extracted, and purified as described (5, 9). $E2_{384-715}$ was expressed and secreted from recombinant CHO cells as described for the truncated $E2_{661}$ (5). For purification of CHO/$E2_{715}$, CHO cells conditioned medium was concentrated 15-fold by ultrafiltration, followed by a further 10-fold volume reduction by ammonium sulfate precipitation at 75% saturation, and redissolution into 25 mM Tris chloride/1 mM EDTA, pH 7.5. The monoclonal antibody (mAb) 5E5/H7 (raised against HeLa E1/E2) was used for purification. The antibody column was equilibrated in 25 mM Tris chloride/0.15 M NaCl, pH 7.5. The ammonium sulfate-precipitated E2 was dissolved in 25 mM Tris chloride/1 mM EDTA, pH 7.5, and loaded onto the column. The column was washed with phosphate-buffered saline (PBS)/1 M NaCl and then eluted with 3–4 column volumes of Actisep (Sterogene, Arcadia, CA). All of the yellow-colored Actisep-containing fractions were pooled, con-

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Abbreviations: HCV, hepatitis C virus; HVR1, hypervariable region 1; E2, envelope glycoprotein 2; MFI, mean fluorescence intensity; NOB, neutralization of binding; mAb, monoclonal antibody.

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centrated in a stirred cell ultrafilter, and diafiltered into PBS buffer. $E2_{384-715}$ was expressed and secreted from recombinant baculovirus infected cells and purified as described (10). For expression and secretion from yeast of $E2_{384-715}$, we used the *Saccharomyces cerevisiae* strain S150-2B and the secretion vector YEpsec1 (11). E2 is secreted as a core glycosylated peptide of 55 kDa. Yeast/$E2_{715}$ was purified by affinity chromatography using a lectin column, and the same procedure was used for purification of baculovirus/E2 (10). After purification, all the HCV envelope proteins were >90% pure. ELISA for all antigens were done according to published procedures (10).

Sera and mAbs. Rabbit antisera were raised against all the envelope proteins described above, and sera from chimpanzees immunized with HeLa E1/E2 or with a combination of yeast/$E1_{190-330}$ and baculovirus/$E2_{404-661}$ (9) were obtained. The mAb 291 (IgG1) was obtained from mice immunized with CHO/$E2_{715}$ and screened for the ability to recognize E2 bound to target cells. A synthetic peptide consisting of HCV-1 amino acids 384–414 [E2 hypervariable region 1 (HVR1)] was coupled through the amino-terminal residue to diphtheria toxoid and used to immunize mice. The mAbs resulting from the fusion were screened by Elisa with overlapping biotinylated 8-mer peptides from amino acid 288–487 on streptavidin-coated plates. An IgG1 mAb (1G2A7) was isolated that recognized the epitope 384–414 in ELISA. For the present study 34 sera from chronic hepatitis patients infected with HCV genotypes 1a, 1b, 2, and 3a (12) were selected.

Binding Assay. MOLT-4 cells (10^5 per well) were pelleted in 96 U-bottom microplates by centrifugation at 200 × *g* for 5 min at 4°C. Twenty microliters of HCV proteins diluted in PBS at different concentrations was mixed with the cell pellet and incubated at 4°C for 1 hr. Nonbound HCV proteins were removed by two centrifugations in PBS at 200 × *g* for 5 min at 4°C. Cells were subsequently incubated for 30 min at 4°C with various dilutions of sera from humans, chimpanzees, or rabbits that had been either infected with HCV or immunized with HCV recombinant proteins; where possible, the corresponding preimmune sera were used as control. The cells were washed twice in PBS and incubated for 30 min with the appropriate dilution of fluorescein isothiocyanate-labeled antiserum (either to human IgG or rabbit IgG). Cells were subsequently washed in PBS at 4°C and resuspended in 100 μl of PBS; cell-bound fluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson) by using the Lysis II software program from Becton Dickinson. This program produces histograms of each cell sample and calculates the mean fluorescence intensity (MFI) of the cell population, which directly relates to the surface density of fluorescently labeled HCV proteins bound to the cells. MFI values of cells incubated with or without HCV proteins and with immune or preimmune sera are compared. The threshold for positivity is set for each experiment by flow cytometric analysis of cells without HCV proteins bound that have been incubated with antisera to HCV proteins and the fluorescein isothiocyanate-labeled second antibody.

Neutralization Assay. Twenty microliters of CHO/$E2_{715}$ (PBS at 0.5 μg/ml) was mixed with various dilutions of sera from humans, chimpanzees, or rabbits that either were infected with HCV or had been immunized with HCV recombinant proteins. After incubation at 4°C for 1 hr, pellets of MOLT-4 cells were added and incubated for 1 hr at 4°C. Nonbound HCV proteins and antibodies were removed by two centrifugations in PBS at 200 × *g* for 5 min at 4°C. Cells were subsequently incubated for 30 min at 4°C with 1/100 dilution of sera from animals (the same species of the neutralizing sera) immunized with HCV-envelope recombinant proteins. Revealing the binding with antibodies from the same species of the neutralizing serum is critical because nonneutralizing anti-E2 antibodies could cover E2 after it is bound to target cells and could, therefore, interfere with assessment of neutralization when the binding was revealed with an anti-E2 serum from a different species. The cells were washed twice in PBS and incubated for 30 min with the appropriate dilutions of fluorescein isothiocyanate-conjugated antiserum to IgG. Cell-bound fluorescence was analyzed as described for the binding assay above.

RESULTS

Binding of E2 to Target Cells Is Measurable and Has High Affinity. We reasoned that HCV envelope proteins should bind to cells susceptible to HCV infection and that this binding should be measurable with antibodies specific for envelope proteins. Indirect immunofluorescence experiments were done to assess the ability of HCV envelope proteins to bind to MOLT-4 cells, a human cell line reported to allow low-level HCV replication *in vitro* (13). Cells were incubated at 4°C with HCV recombinant envelopes (E1/E2 or E2), expressed in either yeast, insect cells, or mammalian cells (HeLa or CHO), and subsequently incubated with polyclonal sera from rabbits immunized with the corresponding recombinant proteins. After incubation with fluorescein isothiocyanate-conjugated antiserum to rabbit IgG, binding of HCV proteins was indirectly detected by flow cytometry as cell-bound fluorescence. The representative experiments in Fig. 1 show that recombinant E1/E2 or E2 expressed in mammalian cells, but not in yeast, can bind human cells, whereas E2 expressed in insect cells has a low, but detectable, binding. Identical data were also obtained when hepatocarcinoma cell lines or freshly purified human B cells were used as target cells, whereas we never found any binding on mouse cell lines or freshly isolated hepatocytes from mice (data not shown).

After incubation of the target cells with increased concentrations of E1/E2 or E2, we found (Fig. 2*A*) that the binding of E2

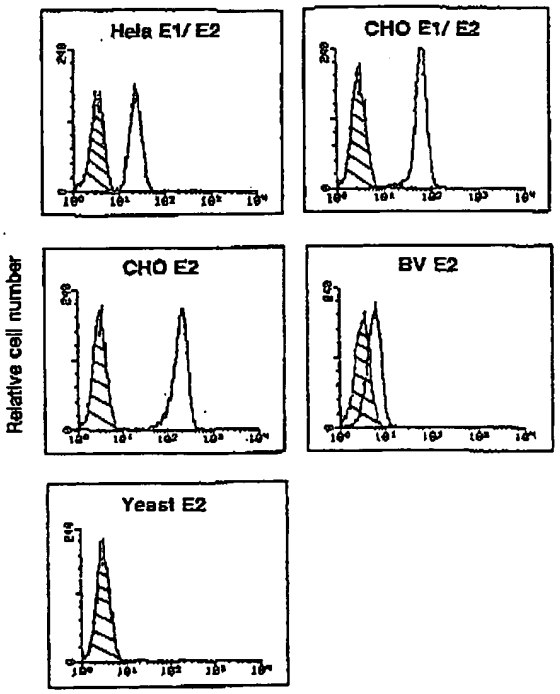

FIG. 1. Differential binding to human cells of HCV recombinant envelopes expressed in various systems. MOLT-4 cells were incubated with medium alone (hatched curves) or the indicated HCV recombinant envelopes at 10 μg/ml (open curves). Staining and flow cytometry analyses were done as described.

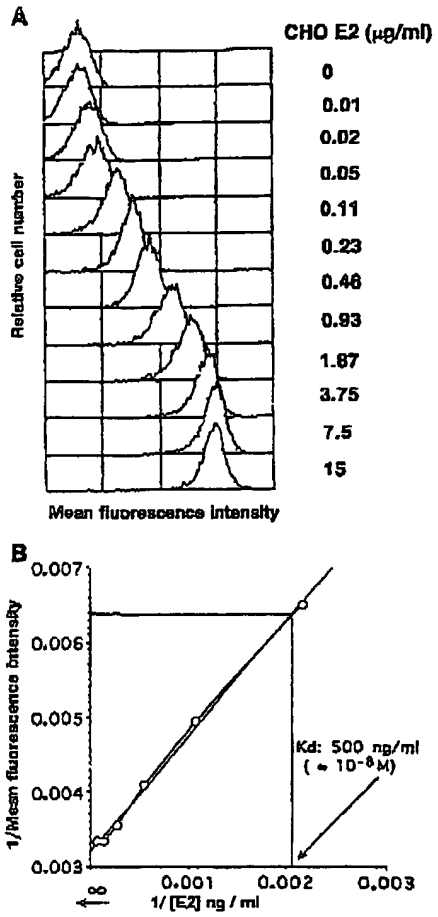

FIG. 2. CHO/E2 binds to human cells with high affinity. MOLT-4 cells were incubated with the CHO/$E2_{715}$ concentrations indicated in *A*. Staining and flow cytometry analyses were done as described. Binding affinity was calculated in *B*. On the *y* axis, net MFI values for each E2 concentration were calculated by subtracting the MFI obtained with rabbit anti-E2 serum and fluorescein isothiocyanate–goat anti-rabbit serum without E2 from that obtained with E2. Net MFI (*y* axis) and E2 concentration (*x* axis) were plotted as reciprocal values.

expressed in mammalian cells reached a plateau at a concentration of ≈10 μg/ml. Because this binding can be saturated, we estimated the affinity of recombinant E2 for its putative receptor by using the double-reciprocal-plot method described (14) for calculation of the affinity of hapten–antibody interaction. In Fig. 2*B*, the estimated affinity is expressed as K_d, and it is equal to the reciprocal of the free E2 concentration at which half the E2 concentration is bound to its putative receptor. We found that the K_d of E2 for target cells is $\sim 10^{-8}$ M. From the above data we conclude that E2 is probably the protein responsible for specific binding of E1/E2 complexes to target cells, although a role for E1 cannot presently be excluded.

Binding Can Be Neutralized with Antibodies to E2 HVR1. Having established a binding assay, we asked whether this binding could be neutralized with antibody to E2. We had made rabbit polyclonal antisera specific for CHO/$E2_{715}$ and assessed their ability to neutralize binding of E2. The protein E2 (at 0.5 μg/ml—i.e., the K_d) was mixed with serial dilutions of the rabbit antisera. The E2/antibody mixture was then incubated with target cells, and the binding of E2 was subsequently determined. We found that sera from rabbit immunized with E2 expressed in mammalian cells can neutralize binding of E2 to target cells (data not shown). For other viruses, epitopes able to induce neutralizing antibodies have been located in regions showing a high degree of variability. We investigated whether a mAb against the HCV-E2 HVR1 (aa 384–414) (15) neutralized binding of E2. Fig. 3 shows that the HVR1-specific mAb can partially neutralize E2 binding. Thus, we conclude that binding of E2 is, at least in part, mediated by this HCV hypervariable region.

Antibodies That Neutralize Binding of E2 Correlate with Protection from Infection. It has been shown that vaccination with recombinant envelope proteins expressed in mammalian cells (HeLa), but not in yeast or insect cells, can protect chimpanzees from primary infection by an homologous HCV isolate (9). To investigate whether the binding and subsequent neutralization of E2 were relevant to the binding of HCV to target cells, we compared the NOB titers in the sera of chimpanzees vaccinated and protected from subsequent challenge with the sera from chimpanzees immunized but susceptible to HCV challenge. Fig. 4 shows that chimpanzees immunized with HeLa E1/E2 all developed NOB antibodies. From the quantitative point of view, chimpanzees with NOB titers of at least 1/600 were protected from primary infection, chimpanzees (no. 470 and WS181) with NOB titers of ≈1/300 developed a mild infection and resolved. Chimpanzees (no. 590 and 635) immunized with envelopes expressed in insect cells or yeast did not have NOB antibodies, were infected, and develop hepatitis; one of them resolved (9). Fig. 4 also shows that ELISA titers to E2-HVR1 were comparable in protected versus nonprotected chimpanzees. Thus, we conclude that high titers of NOB antibodies are associated with protection from infection and that neutralization induced by vaccination does not depend on antibodies to the HVR1.

HCV Infection Elicits Low or No Neutralizing Antibodies. We then assessed the presence of neutralizing antibodies in the sera of patients chronically infected with various HCV genotypes (12). The data in Table 1 demonstrate that HCV infection does not elicit neutralizing antibodies in 60% of cases, although low titers of neutralizing antibodies were

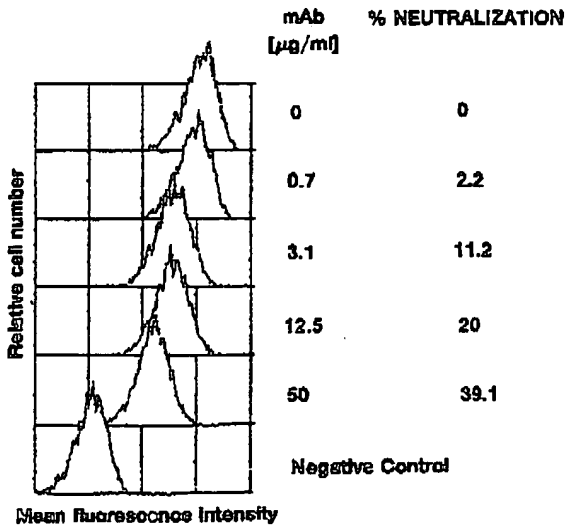

FIG. 3. Antibodies to HVR1 neutralize binding of E2. CHO/E2 was preincubated with the indicated concentrations of purified mAb (1G2A7) specific for HCV-E2 HCVR1. Antibody/E2 mixture was then incubated with MOLT-4 cells, and binding was revealed by using mAb 291, which recognizes E2 bound to target cells. MFI values without neutralizing mAb (positive control), without E2 (negative control), and with E2–antibody complexes (experimental values) were measured, and specific neutralization was determined according to the following equation: Specific neutralization = [(positive control MFI − experimental MFI)/(positive control MFI − negative control MFI)] × 100.

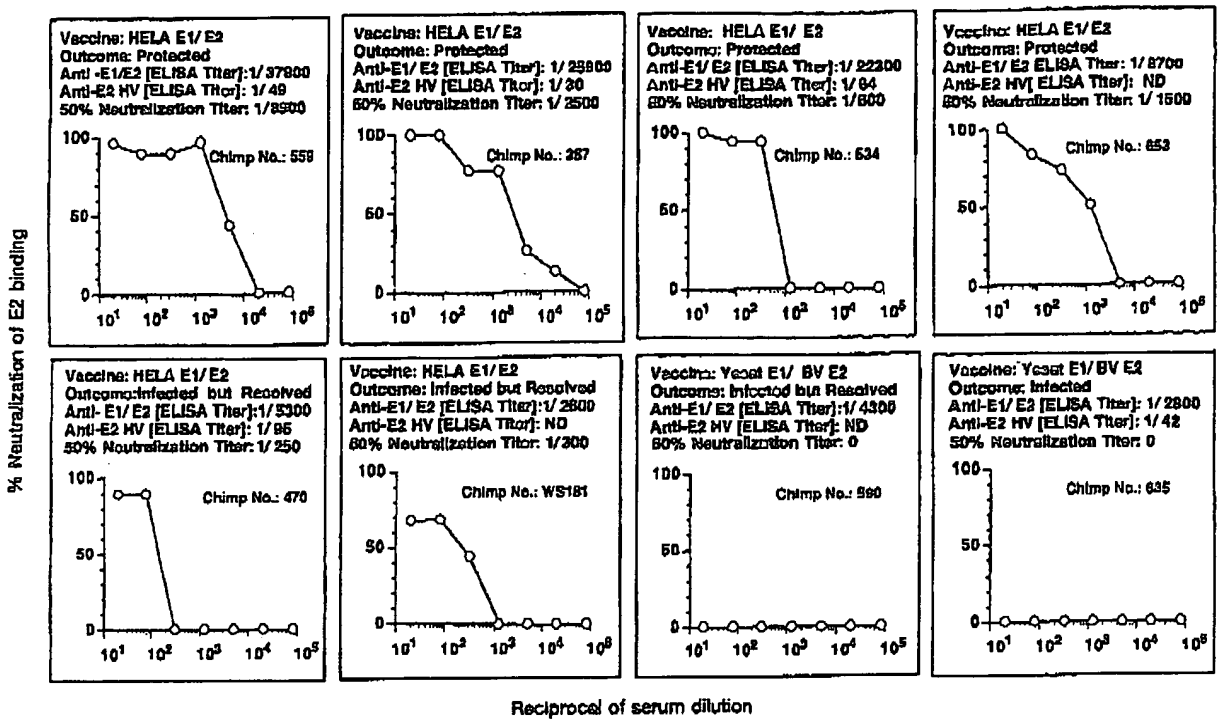

FIG. 4. Antibodies that neutralize binding of HCV envelope correlate with protection from infection. Serial dilutions of sera from chimpanzees vaccinated with recombinant envelope proteins (9) were tested for their ability to neutralize binding of E2. Each square indicates the envelope proteins used as vaccine, the outcome of challenge with HCV-1-containing plasma, the ELISA titers against both HeLa E1/E2 and the peptide corresponding to E2-HVR1, and the neutralization calculated as in Fig. 3 and expressed as 50% neutralization titers.

detectable in the remaining infections. Interestingly, Table 1 shows that the ability to neutralize binding of E2 from the HCV-1 genotype is not specific to infection by the homologous HCV-1 genotype because the low titers of neutralizing antibodies are equally distributed among sera from patients infected with various genotypes. Also in chimpanzees persistently infected with the HCV-1a genotype, we found low titers of neutralizing antibodies in only one-eighth of the cases (data not shown).

Because patients with chronic hepatitis have low or no NOB antibody titers, we asked whether neutralizing antibodies played a role in the benign outcome of HCV infection. Therefore, we assessed NOB titers in the sera of seven infected individuals with a benign clinical course—i.e., healthy seropositives (16). In this case also we found the absence or low levels of neutralizing antibodies (data not shown).

We conclude that HCV infection elicits low or no neutralizing antibodies regardless of the clinical outcome of the infection or of the infecting genotype.

DISCUSSION

We set up a cytofluorimetric test to assess the specific binding of HCV envelope proteins to cell lines as well as to freshly isolated cells. We have found that E2 binds to putative target cells with a fairly high affinity ($K_d \approx 10^{-8}$ M), although we cannot exclude that E1 has a role in the binding of the whole viral particles. The finding that HCV-E2 binds with high affinity to target cells only when expressed in mammalian cells is most likely due to the "physiologic" N-glycosylation and conformation it undergoes when expressed in mammalian cells.

We have found that it is possible to quantitatively assess the neutralization of E2 binding (NOB) by antibodies. Because the first contact between a virus and its host cell occurs via binding of the virus envelope to cell-surface receptors, neutralization of this interaction is expected to be one of the major infection-neutralization modes. A key question is whether E2-binding neutralization *in vitro* has relevance to neutralization of infection *in vivo*. It has been shown that vaccination with recombinant envelope proteins expressed in mammalian cells, but not in yeast or insect cells, can protect chimpanzees from primary infection by an homologous HCV isolate (9), and this parallels our finding that HCV recombinant envelope proteins bind to target cells with high affinity only when expressed in mammalian cells. Moreover, we have found a direct correlation between NOB antibody titers and protection from infection in vaccinated chimpanzees. All the chimpanzees immunized with Hela E1/E2 developed NOB antibodies, but only those chimpanzees with neutralizing titers of at least 1/600 were protected from primary infection, whereas chimpanzees with titers of ~1/300 were infected, although they resolved quickly. The two chimpanzees immunized with envelopes expressed in insect cells or yeast did not have NOB antibodies, and both were infected after challenge. The finding that one of them (no. 590) was infected but then resolved, indicates that neutralizing antibodies are critical to protection from infection, but once the infection is established and the virus is mostly intracellular, cell-mediated immunity should play a major role in protecting from the disease (16).

Although protection induced by vaccination does not depend on antibodies to the HVR1, we have also found that antibodies to HVR1 can neutralize E2 binding. This result confirms recent data (8) and demonstrates that at least two neutralizing epitopes, one of which is hypervariable, should exist on the E2 protein.

NOB antibodies were absent in 60% of sera from patients chronically infected by HCV. In those patients who had measurable serum-neutralizing activity, the titers were in all

Table 1. Neutralizing activity in sera from chronic hepatitis patients infected with different HCV genotypes

Patient	Genotype*	Anti-E2†	Neutralization‡
2BD	1a	1/1230	No
6BD	1a	1/10	No
12BD	1a	1/630	No
13BD	1a	1/50	No
FJG	1a	1/500	No
FS	1a	1/4330	++
GHH	1a	1/2290	No
LHM	1a	1/3630	No
NS	1a	1/640	+
TAP	1a	1/8250	+
TFW	1a	1/11340	++
1BD	1b	1/12000	No
RCR	1b	1/640	No
SA	2	1/380	No
4BD	2	1/2020	++
RA	2	1/5340	++
RLE	2	1/1510	No
ERF	2	1/1210	No
GB1305Y	2	1/5310	++
GB1289P	2	1/3340	+
GB1164R	2	1/4880	++
GB1147A	2	1/670	No
GB1136N	2	1/520	+
3BD	3a	1/1560	No
7BD	3a	1/1460	No
JGL	3a	1/160	No
GB1414C	3a	1/6200	++
GB1328E	3a	1/2380	++
GB1332V	3a	1/1260	+
GB1228A	3a	0	No
GB1400V	3a	1/1410	+
GB1222L	3a	1/360	No
GB1387F	3a	1/670	No
GB1407R	3a	1/1900	No

*Genotyping of patients' sera was done as described (12).

†Serum antibody titers specific for CHO/E2$_{715}$ were assessed by ELISA.

‡Neutralization of E2 binding was assessed as described. The two crosses indicate 20–40% neutralization at serum dilution between 1/80 and 1/200. The single cross indicates 10–20% neutralization at serum dilution between 1/40 and 1/80. No, absence of neutralizing activity.

cases very low, and we could not assess 50% neutralizing activity. Because E2 shows considerable sequence heterogeneity among HCV isolates, one could speculate that neutralizing antibodies in patients are present but recognize variable regions of the E2 proteins. Such antibodies may not be detected in binding assays performed with recombinant E2 from HCV isolates different from the patients' isolates. To investigate this possibility, we assessed the presence of neutralizing antibodies in the sera of patients chronically infected with various HCV genotypes. Because in the subset of patients who had detectable NOB antibodies, the presence of the neutralizing activity was not specific to infection with the homologous HCV-1 genotype, we favor the possibility that NOB antibodies are, at least in part, directed to conserved regions of E2. Should neutralizing antibodies induced by vaccination have the same specificity as NOB antibodies induced by infection, one could speculate that cross-neutralization of infection may be achievable by high titers of neutralizing antibodies induced by vaccination.

There was no difference in titers of NOB antibodies between chronically infected patients and "healthy seropositives." In both cases neutralizing antibodies are induced after infection is established, when the virus is mostly intracellular. In analogy to the situation observed with the immunized chimpanzees who got infected, it is likely that the different disease outcomes may be related to the individual cell-mediated immune response (16).

In conclusion, the NOB test we have developed is a fast and high-throughput assay that measures neutralization of binding of recombinant HCV-E2. The main findings that come from the present study are as follows: (*i*) HCV-E2 can bind target cells with high affinity, suggesting it should be responsible for HCV binding to host cells; (*ii*) high NOB titers correlate with neutralization of infection in chimpanzees; (*iii*) vaccination can induce high titers of NOB antibodies, whereas in infection NOB antibodies are low or absent; (*iv*) At least two neutralizing epitopes, one conserved and one variable, should exist on the E2 protein.

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